# A Comparison of Western Blotting Techniques Using Phospho-Specific Antibody Detection of Protein Kinase Activation


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*Western blotting is a popular method for using highly selective antibodies to visualize proteins in cell or tissue extracts. Indeed, the availability of specific antibodies that selectively target post-translational modifications (e.g., phosphorylation) of the protein of interest can provide a highly sensitive and non-radioactive measure of protein kinase activation via Western analysis. In this article the detection of MAP kinase activation using Promega's Anti-ACTIVE™ MAPK pAb and prequalified HRP- and AP-labeled secondary antibodies is presented to illustrate commonly used Western methods.*

## INTRODUCTION

For the past 20 years, Western blotting has been used as a simple and effective procedure for detecting proteins following separation by denaturing gel electrophoresis (1). More recently, numerous advances in the ease and efficiency of Western blotting have improved the speed and accuracy of this method. For example, the advent of minigels ten years ago greatly reduced the time required for electrophoretic separation (2). Similarly, the development of semi-dry protein transfer methods also has reduced the time required for this step (3), although in certain applications the 'wet' transfer method can provide greater uniformity of transfer. A wide variety of options have also been developed for membranes (e.g., nitrocellulose and nylon-based), blocking solutions, and detection strategies including both radioactive (e.g., $^{125}$I-Protein A) and non-radioactive (via chemiluminescent or colorimetric detection) reagents and methods (4,5). Finally, advances in visualization using high-contrast, high-sensitivity films (e.g., Kodak® BioMax® chemiluminescent film) or imaging instrumentation capable of integrating a range of

signals (e.g., BioRad's Fluor-S Imager®) provide more accurate and permanent records of immunoblot data. More detailed descriptions of the theory and methodology of Western detection can be found in numerous reviews (e.g., 6-8). In addition, specific techniques are often described in the literature supplied by the many vendors of Western blotting equipment or reagents.

Despite the numerous advances made toward optimizing Western blotting procedures, this method remains in many respects an 'art', given the many variables and the extent to which the optimum conditions can vary from one sample to the next (9). Indeed, there can be considerable variability in the recovery of different proteins from cells or tissues, the rate and degree of transfer to the membrane support, the affinity of proteins for the membrane, and the extent to which antibody recognition of a particular protein is masked by the presence of other proteins in the extract (10,11). Antibodies also differ in the degree to which recognition of the targeted peptide sequence(s) is dependent on the conformation of the epitope. In cases where the native form of the protein is preferred, proteins that have been separated under denaturing conditions can sometimes be sufficiently refolded to allow recognition by conformation-specific antibodies (12). In contrast, some denatured proteins (e.g., G-protein gamma subunits) must be transferred to the membrane support under more stringent conditions to prevent spontaneous refolding (13). Finally, the effectiveness of different blocking agents can vary considerably with both the amount and nature of a particular extract being analyzed and the specific characteristics of the primary and secondary antibody used to probe the blot (4).

This article describes several commonly used approaches for Western blotting. The data provided were generated using Promega's Anti-ACTIVE™ MAPK pAb, a rabbit polyclonal antibody selective for the dually phosphorylated, active form of the target enzyme (14,15). In addition, the value of using high quality HRP- or AP-conjugated secondary antibodies in generating optimum signal-to-noise ratios is illustrated.

## REAGENTS AND PROTOCOLS

### General reagents

IgG-free and protease-free BSA (Jackson Laboratories), RPMI 1640 medium (Gibco®-BRL), horse serum (Bio-Whittaker), fetal bovine serum (HyClone), Nerve Growth Factor (NGF; Promega Cat.# G5141). All other chemicals were of the highest grade and obtained from Fisher Scientific or Sigma Chemical Company.

### Antibodies

Anti-ACTIVE™ MAPK pAb (Promega Cat.# V6671) is a multi-step affinity purified rabbit polyclonal antibody made against a dually phosphorylated peptide that corresponds to the active form of the ERK1 and ERK2 enzymes (14,15). The Anti-ACTIVE™ series of antibodies, which also includes Anti-ACTIVE™ JNK (Cat.# V7931 and V7932) and Anti-ACTIVE™ p38 (Cat.# V2901 and V2902), targets the highly conserved threonine-X-tyrosine (Thr-X-Tyr) motif in the 'phosphorylation lip' of each ERK/MAP kinase superfamily member (8). Anti-ACTIVE™ MAPK pAb is supplied in 0.01M sodium phosphate, 0.02M NaCl (pH 7.4), and does not include any preservatives. Promega's Donkey Anti-Rabbit IgG (H + L) secondary antibodies, which are prequalified for use with the Anti-ACTIVE™ pAbs and crude cell extracts, are available as both an HRP- (Cat.# V7951) and an AP-conjugate (Cat.# V7971).

### Preparation of extracts from NGF-stimulated cells

PC12 (rat phaeochromocytoma) cells were grown in RPMI 1640 medium with 10% horse serum, 5% fetal bovine serum and 15mM HEPES in 150mm dishes coated with rat tail collagen type I (6µg/cm$^2$). Cells were grown to near confluence and then exposed to fresh medium containing serum and 0.5mM EGTA for approximately 18 hours prior to treatment with 50ng/ml NGF for 5 minutes at 37°C. Cells then were washed once with cold phosphate-buffered saline and extracts were prepared as previously described (16). Briefly, cells were scraped into iced homogenization solution (20mM Tris-HCl [pH 7.5] containing 20mM p-nitrophenyl phosphate as a general phosphatase inhibitor, 1mM EGTA as a $Ca^{2+}$ chelator, 50mM sodium fluoride as a Ser/Thr phosphatase inhibitor, 50µM sodium orthovanadate as a Tyr phosphatase inhibitor and 5mM benzamidine as a protease inhibitor) and disrupted on ice using 120 strokes of a tight fitting Dounce homogenizer. All subsequent steps were performed at 4°C. The resulting homogenates were centrifuged at 4,000 x *g* for 5 minutes and the supernatants were collected and pooled. Supernatants from a second centrifugation step (97,000 x *g* for 60 minutes) were collected and frozen as aliquots in liquid nitrogen. Final aliquots were stored at -80°C, where they were stable for 6-12 months.

## DISCONTINUOUS SDS-PAGE AND ELECTROPHORETIC TRANSFER

Precast 12 well, 10% SDS-PAGE gels (Novex) were rinsed with NANOpure® water to remove the azide storage buffer and then assembled into a discontinuous slab gel apparatus. In a microcentrifuge tube, protein samples (2µg/lane) were mixed with 2X Sample Buffer (Novex) containing 20mM DTT as the reducing agent. Extract samples were heated for 2 minutes at 95°C and centrifuged for 5 seconds in a microcentrifuge. Samples (15µl) or prestained molecular weight markers (5µl, Novex) were loaded into each lane and the gel was run at 125V (constant voltage) until the dye front reached the end of the gel (~1.5-2 hours; 17). The gel was soaked in transfer buffer (0.15M glycine, 20mM Tris, 20% methanol at 4°C) for 15 minutes. The proteins were transferred to either a nitrocellulose (0.45µm, BioRad) or PVDF (0.45µm, Millipore®) membrane measuring approximately 60 x 80mm (the nitrocellulose membrane was soaked in transfer buffer for 15 minutes to ensure that it was fully wet before transfer).

The electroblotting unit was assembled according to the manufacturer's instructions, cold (4°C) transfer buffer was added to the apparatus and the proteins transferred to the membrane at 100V for 1 hour at 4°C.

## WESTERN BLOTTING

The procedures were performed as described in Figure 1A (nitrocellulose) or Figure 1B (PVDF). The composition of TBST was TBS/0.05% Tween® 20. The composition of the PVDF Buffer was TBS/0.2% I-Block™/0.1% Tween® 20. I-Block™ (Tropix) is a highly purified preparation of casein, prequalified for use with Western blotting applications.

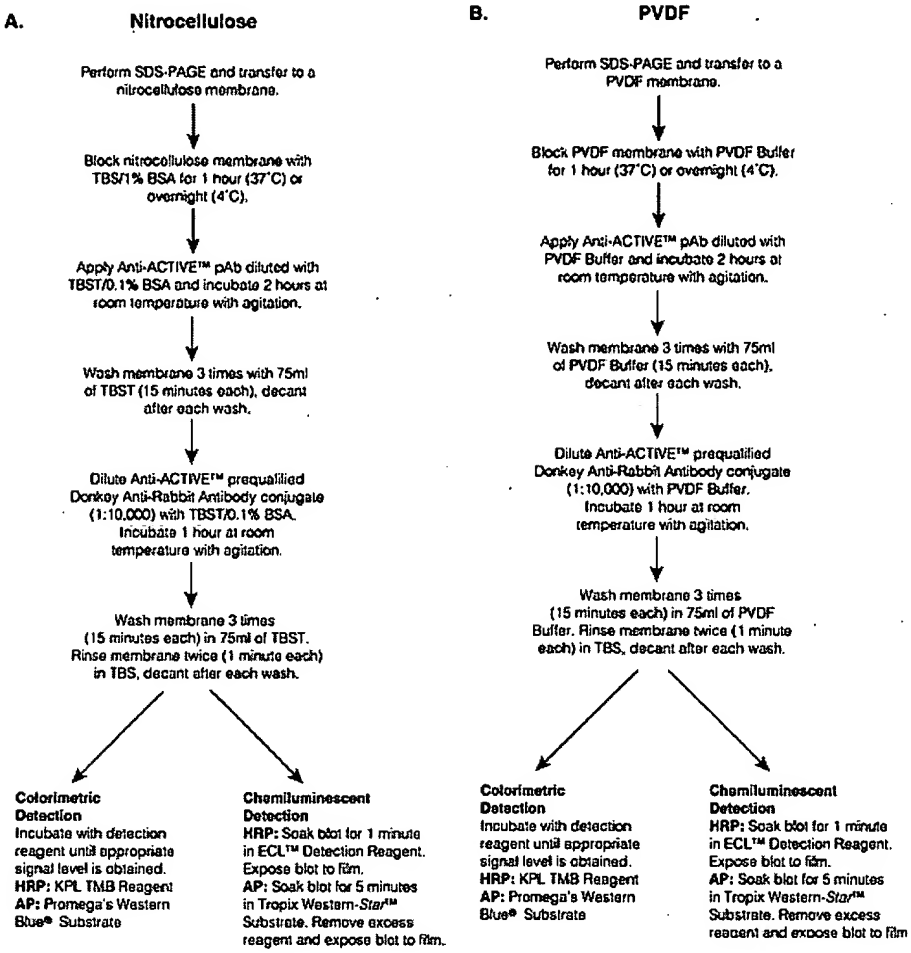


**Figure 1. Overview of Western blotting protocols illustrating the use of different membranes, blocking agents and detection methods. Panel A:** Protocols for use with nitrocellulose membranes. **Panel B:** Protocols for use with PVDF membranes. Recommended dilutions of the Anti-ACTIVE™ pAbs are 1:20,000 for Anti-ACTIVE™ MAPK pAb, 1:5,000 for Anti-ACTIVE™ JNK, 1:2,000 for Anti-ACTIVE™ p38 and 1:10,000 for the Donkey Anti-Rabbit IgG (H + L) secondary antibodies (both the HRP- and the AP-conjugated). Note that it may be necessary to empirically determine the optimum dilutions for your system.

## ALKALINE PHOSPHATASE (AP)-MEDIATED CHEMILUMINESCENT DETECTION

The blots were developed using the Tropix Western-*Star*™ Kit. Following the transfer step, the blots were washed twice for 2 minutes each with 10ml of Tropix Assay Buffer (diluted to 1X from the 10X stock with NANOpure® water) and then covered with a thin layer (~0.75ml per blot) of substrate solution. For PVDF membranes, the CDP-*Star*™ ready-to-use substrate solution was used. For nitrocellulose membranes, the substrate solution was prepared by mixing 37.5µl of Nitro-Block™-II (Tropix) with 0.75ml of the CDP-*Star*™ ready-to-use substrate solution. After a 5-minute incubation at room temperature, the substrate solution was drained and the blots were placed between two paper towels and gentle pressure applied to remove excess solution. The membranes then were placed between two sheets of plastic wrap and exposed to film (Kodak® BioMax® chemiluminescence film) for 30 seconds.

## PEROXIDASE (HRP)-MEDIATED CHEMILUMINESCENT DETECTION

The blots were developed using the ECL™ Chemiluminescent Detection Reagents (Amersham). The detection solution was prepared by mixing equal volumes of Solution 1 with Solution 2 to yield sufficient volume to cover the membrane (~0.125ml/cm$^2$ of membrane, 1ml total). Immediately after mixing, the solution was poured directly onto the protein containing side of the blot surface and incubated for 1 minute at room temperature. The blot then was removed from the Detection Solution and excess solution removed by touching the edge of the blot against a piece of tissue paper. The blot was enclosed in plastic wrap and exposed to film (Kodak® BioMax® chemiluminescence film) for 30 seconds.

### RESULTS AND DISCUSSION

Among the many options available for performing Western blotting, the choice of membrane type and the detection system used are parameters that are commonly varied. Figure 1 presents protocols for several commonly used approaches. Figure 1A describes the use of nitrocellulose membranes while Figure 1B outlines the steps used with PVDF (nylon-based) membranes. These protocols also describe the use of both alkaline phosphatase (AP) and horseradish peroxidase (HRP) conjugated secondary antibodies, and detection using either colorimetric or chemiluminescent methods. While these protocols were developed for use with Promega's Anti-ACTIVE™ pAbs and prequalified secondary antibodies, they also serve as a general guideline for performing and optimizing Western blots.

## COMPARISON OF WESTERN BLOTTING CONDITIONS

To illustrate the use of the protocols presented in Figure 1, we generated data using an experimental system where pheochromocytoma (PC12) cells growing in culture were stimulated with nerve growth factor (NGF) to activate the mitogen-activated protein kinase (MAPK) subfamily of enzymes (e.g., ERK1 and ERK2; reviewed in 18). Detection of ERK1 and ERK2 activation was achieved using a polyclonal antibody (Anti-ACTIVE™ MAPK pAb) that recognizes the dually phosphorylated, active form of these enzymes (14,15).

As shown in Figure 2, we analyzed crude cell extracts prepared from either untreated or NGF-treated PC12 cells. Extract samples were separated by SDS-PAGE under reducing conditions and electroblotted onto either nitrocellulose or PVDF membranes. The blots were probed with the Anti-ACTIVE™ MAPK pAb, followed by incubation with either an AP- (Figure 2A) or HRP- (Figure 2B) conjugated Donkey Anti-Rabbit secondary antibody. Signals then were generated using the appropriate chemiluminescent detection system. As expected, no signal was detected with extracts from untreated PC12 cells, but NGF treatment resulted in strong induction of ERK1 and ERK2. The intensity of the signals obtained with these crude extracts were comparable to those obtained with ~500pg of recombinant ERK2 enzyme (data not shown), illustrating the high-sensitivity achieved by the combined use of the Anti-ACTIVE™ MAPK pAb and the prequalified secondary antibodies. Similar results have been generated using colorimetric detection with the AP-conjugated Donkey Anti-Rabbit secondary antibody and Promega's Western Blue® Stabilized Substrate (Cat.# W3960; data not shown), as well as with $^{125}$I-Protein A in combination with autoradiography (19).

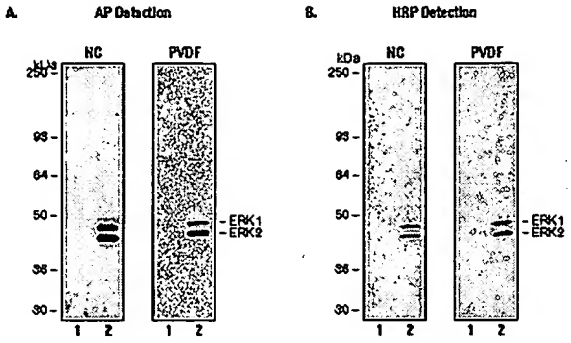


**Figure 2. Comparison of Western blotting protocols.** PC12 cells were treated with NGF, cell extracts prepared and proteins separated as described under Reagents and protocols. The separated proteins were transferred to the indicated membranes and Western blotting performed as described in Figure 1 and below. **Panel A:** Blots were probed with Anti-ACTIVE™ MAPK pAb (1:20,000 dilution) and Promega's Donkey Anti-Rabbit IgG AP (alkaline phosphatase)-conjugated secondary antibody. Detection was performed using the Western-*Star*™ Chemiluminescence Kit. Lanes 1, unstimulated cell extract; lanes 2, NGF-stimulated extracts. **Panel B:** As described in Panel A except Promega's Donkey Anti-Rabbit IgG HRP (horseradish peroxidase)-conjugated secondary antibody was used and detection was performed using the ECL™ Chemiluminescence Detection Reagents. Lanes 1, unstimulated cell extract; Lanes 2, NGF-stimulated extracts.

It is important to emphasize that the washing steps represent a critical element of these Western detection methods. The number of washes, the duration, and the volumes used for each step are important factors in minimizing general background as well as the appearance of nonspecific protein bands. In addition, further improvement of signal-to-noise ratios can be accomplished by systematic adjustments to such variables as the source and percentage of blocking reagent (e.g., 1-5%), the amount of extract run on the gel and the amount of primary or secondary antibody used. This process is highly empirical and illustrates the less predictable, more 'art' driven elements of Western blotting (4,9).

## EVALUATION OF SECONDARY ANTIBODY PERFORMANCE

It has been our experience that the quality and reproducibility of many commercially available secondary conjugates vary among different vendors, as well as between different lots from the same vendor. Based on this observation, we chose to develop our own prequalified secondary antibody reagents for use with the Anti-ACTIVE™ pAb product line. Promega's Donkey Anti-Rabbit IgG secondary antibodies, used in Figure 2, have been selected to provide low nonspecific binding with bacterial or mammalian cell extracts. These antibody preparations have also been optimized to provide minimum cross-reactivity with IgGs from other species (e.g., mouse, sheep, goat, etc.).

As an example of the performance variability of commercial secondary antibody preparations, we compared two commercially available affinity-purified AP-labeled preparations (designated as Vendor X and Y) to Promega's Anti-ACTIVE™ prequalified Donkey Anti-Rabbit IgG pAb AP conjugate. Results were again generated using crude cell extracts prepared from either untreated or NGF-treated PC12 cells. As illustrated in Figure 3, the two antibody preparations from Vendors X and Y (Panels A and B) resulted in significantly more nonspecific binding (seen as diffuse staining and bands other than ERK1 and ERK2 proteins) compared to the Promega secondary antibody conjugate (Panel C). All antibodies were used at the working dilution recommended by the vendor.

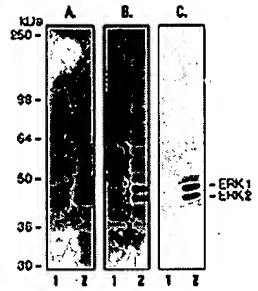


**Figure 3. Comparison of two commercially available secondary antibodies to Promega's Anti-ACTIVE™ pAb prequalified AP conjugate.** PC12 cells were treated with NGF, cell extracts prepared, and proteins separated as described in Reagents and protocols. Proteins were transferred to nitrocellulose membranes and Western blotting performed as described in Figures 1 and 2. The primary antibody was Anti-ACTIVE™ MAPK pAb diluted 1:20,000. Secondary antibodies were as follows: **Panel A**, anti-rabbit AP-conjugate from Vendor X; **Panel B**, anti-rabbit AP-conjugate from Vendor Y; **Panel C**, Promega's prequalified Donkey Anti-Rabbit IgG AP-conjugate. All of the secondary antibodies are affinity-purified preparations that were used at a 1:10,000 dilution. Lanes 1, 2µg of unstimulated PC12 cell extract; lanes 2, 2µg of NGF-stimulated PC12 extract.

## ACKNOWLEDGEMENTS

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## TIPS FOR WESTERN BLOTTING

**Blocking:** To block nonspecific sites on the membrane, incubate blots with the blocking buffer [e.g., TBST (TBS/0.05% Tween® 20/1.0% BSA)] in a shallow plastic dish (e.g., pipette tip box cover) for 1 hour at room temperature. We have found that blots can be soaked in blocking buffer overnight or up to 48 hours at 4°C with similar results.

**Primary antibody:** Use Antibody Sample Buffer (e.g.,TBS/0.05% Tween® 20/0.1% BSA), 20ml per mini-blot] to dilute the primary antibody. Process only one blot per container.

**Secondary antibody:** Dilute the secondary antibody in Antibody Sample Buffer (above). Performing a control blot where the primary antibody is purposely omitted is very useful for identifying whether nonspecific signals are due to the secondary antibody conjugate or the primary antibody.

**Washes:** All wash steps are critical for reducing general background signal and nonspecific binding to discrete bands. If high background is a problem, the number, length and composition of the washes can all be increased.

**General:** Handle miniblots carefully with forceps to minimize problems with nonspecific signals. Wear gloves for all steps to prevent hand contact with film, membranes or detection reagents.

**AP-mediated chemiluminescent detection (Western-*Star*™ Kit):** When developing the blot, drain excess CDP-*Star*™ Solution and place blots between 2 paper towels and press gently to remove any excess liquid. If excess solution is not removed, it can result in high backgrounds. Develop film and prepare additional exposures based on the signals obtained. Signals generated with the Tropix Western-*Star*™ Kit will typically reach a stable plateau within 1-2 hours, followed by a gradual decay in the signal over several hours. Note that the actual initial rate and the stability of the resulting signal are dependent on the absolute intensity - the more intense the signal, the more rapidly the chemicals required for signal development are exhausted.

**HRP-mediated chemiluminescent detection (ECL™):** With this method it is necessary to work quickly once the blots have been exposed to the detection solution; therefore, it is helpful to conduct all steps in the dark room to minimize delays. Do not allow the blots to dry out. After the blots have been incubated with the Detection Solution, drain off excess solution by holding the blot vertical and touching the edge of the blot against a piece of tissue paper. (Do not remove the excess solution by placing the blot between two paper towels as described for the Western-*Star*™ Kit.) Gently place the blot, protein

side down, onto a piece of plastic wrap. Fold over the surrounding plastic wrap to form an envelope that encloses the blot. Avoid applying excess pressure as this can cause high backgrounds. Gently smooth out the air pockets, again, avoiding excess pressure. Place the blot, protein side up and surrounded by plastic wrap, into a film cassette making sure that no Detection Solution leaks out into the cassette. Develop the film and prepare additional exposures based on the signals obtained. Signals generated with the ECL™ Chemiluminescent Detection Reagent will demonstrate 'flash kinetics' reaching a peak signal within 5-10 minutes, followed by a rapid decay. Note that the signal will continue to develop even after removing the blots from the development solution. Do not expose the blot for too long because the background will become darker with time.

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